RESEARCH PLAN PROPOSAL

ISOLATION AND CHARACTERIZATION OF CHLORPYRIFOS DEGRADING MICROORGANISMS AND THEIR POSSIBLE PLANT GROWTH PROMOTING EFFICACY

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INTRODUCTION

India is primarily an agriculture based country with more than 60-70 per cent of its population dependent on agriculture. India's fast growing population is projected to cross 1.3 billion by 2020 (Kanekar *et al*., 2004). Indian pesticide market is second largest in Asia which ranks 12th globally with a net production of 82,000-85,000 million tons. Out of which 43,000-85,000 tons of pesticides are being used to control various crop pests (Subramanyam *et al.,* 2012).

In recent years, plant protection has become one of the essential inputs in crop production. In the context of changing cropping patterns, introduction of high yielding varieties, application of high doses of fertilizers, with enhanced irrigation facilities, pests have assumed a special significance and more and more pesticides are being applied (Anuja, 2005)

Application of the pesticides on agricultural crop is now a common practice and is an important factor of integrated pest management (IPM) strategies. It adversely affects the properties of the soil as well as it alters the pH of the soil required for microbial activities of beneficial bacteria to act upon (Rahman and Motoyama 2000; Maliwonski 2000). Large complex of insect pests ranging from borers to root feeding insects are responsible for heavy losses of commercial crops, both quantitatively and qualitatively. In order to reap maximum yields, the farmers resort to pesticide application to combat the pest problem (Anuja, 2005).

The effect of pesticides on microbial communities was variable with pesticide types, their doses and field conditions (Bakalivanov, 1990; Cernakova 1993). Pesticide exposure inflicts chronic and acute threats to human health. For example, long term low dose exposure to pesticide causes immune suppression, hormonal disruption, diminished intelligence, reproductive abnormalities and carcinoma (Gupta, 2004).

Amongst the most important problems associated with pesticide application are their possible persistence in the environment and therefore, their possible incorporation into the food chain affects ecosystem and human beings (Liu and Xiong, 2001). Due to environmental significance of pesticides and their residues, a thorough understanding of the physical, chemical and biological forces acting upon these chemicals is important (Xuang *et al.,* 2000). Approximately, less than 0.1% of applied pesticides reach the target pest, leaving the bulk to affect the

environment (Ardley, 1999).

The indiscriminate use of pesticides has led to widespread contamination of water, air and soil with pesticide residues. These include Monocrotophus- 10700 million tons (MT), acephate -6400 MT, and Chlorpyrifos -5000 MT (Bhagobaty and Malik, 2008).

Organophosphorus pesticides (OPPs) are widely used pesticides or chemical warfare agents due to their high toxicity towards insects, mammals and other animals and their extensive use pesticides contaminate the environment. The risk of its percolation in soil increases when pesticide is not degraded and biological persistence poses health risk to humans. Chlorpyrifos is an organophosphorus insecticide widely used across the world. It is used for controlling pests during the production of agricultural commodities and for treating the soil in which different crops are grown. It is a broad spectrum pesticide with trade names Dursban, Lorsban and Spannit, displaying insecticidal activity against a wide range of insect and arthropod pests.

Chlorpyrifos-oxon and 3, 5, 6-trichloro-2-pyridinol (TCP), the two potent degradation products of chlorpyrifos, have been found in groundwater and are very much stable. In soil, chlorpyrifos may remain biologically active for periods ranging from days to months. Dosage rates, soil type, soil moisture and organic matter content, temperature and insecticide formulation are among the factors which influence biological persistence (Read, 1976; Tashiro and Kuhr, 1978). Many methods for the detection of chlorpyrifos residues in the environment, including in water, soil and some fruits and vegetables, have been reported, but most of these methods are based on multi residue analysis, and most studies investigated chlorpyrifos and other pesticides but did not focus on the metabolites of these pesticides, such as the toxic metabolite of chlorpyrifos 3, 5, 6-trichloro-2-pyridinol-TCP (Yucel *et al.,* 1999).

Many chemical and enzymatic methods for remediation of pesticide contaminated soils have been employed. But, none of them gave satisfactory results. Microorganisms being the cheapest and easily available sources are most readily used for remediation of contaminated soils. Bioremediation which includes the gainful utilization of microorganism for the metabolism or biodegradation of target pollutants into safer and innocuous products is amongst the potent technologies that are being used globally for the restoration of contaminated sites.

Insecticides and their degradation products, generally get accumulated in the top soil and influence not only the population of various groups of soil microbes but also their biochemical activities like nitrification, ammonification, decomposition of organic matter and nitrogen fixation (Agnihotri *et al*., 1981). Microorganisms play an important role in degrading synthetic chemicals in soil (Alexander, 1981).

In soil ecosystem, microorganisms including Plant growth promoting Rhizobacteria (PGPR) participate in the myriad of recycling processes of nutrients to sustain the soil fertility (Woyessa and Assefa, 2011). Soil microorganisms, specifically PGPR facilitate plant growth by: 1) solubilizing insoluble phosphates; 2) fixing atmospheric nitrogen and transporting it to plants; 3) facilitating uptake of other plant nutrients and 4) synthesizing siderophores and phytohormones (yadav *et al.,* 2011; Zaidi *et al.,* 2009; Saber *et al.,* 2009). Since soil microorganism are very sensitive to environmental change and a significant degradation of the microbial community can occur following drastic disturbance, both in terms of biomass and species composition (Lin *et al.,* 2007).

REVIEW OF LITERATURE

Pesticide is a general term for substances, which are used to poison pests (weeds, insects, rodents etc.). Synthetic pesticides have been popular with farmers, because of their widespread availability, simplicity in application, efficacy and economic returns. But they also have huge environmental costs.

Pesticide consumption in India-

After India's Green Revolution started, the consumption of pesticides in India has increased several hundred folds, from 154 MT in 1954 to 88,000 MT in 2000-2001. According to industry estimates, the pesticide use has high growth potential in India, as the use of agricultural pesticides is markedly low at 0.54 kg /ha as against 3.7 kg/ha in USA and 2.7 kg/ha in Europe. Notwithstanding the fact that overall consumption of pesticides in India as a whole is low than that used in the developed countries of the world, there is still a widespread contamination of water, soil and air with pesticide residues. In India, among different states maximum consumption of pesticides-1999-2000 was in Uttar Pradesh (7459 MT) followed by Punjab (6972 MT), Haryana (5025 MT), Andhra Pradesh (4054 MT), Gujarat (3646 MT). Leading pesticides used in India include monocrotophos (10700 MT- highest consumed), acephate (6400MT), endosulfan (5600 MT) and chlorpyrifos (5000 MT - fourth highest consumed).
(Pesticide Information, Volume XXVIII, No. 3, October- December 2002).

The production of pesticides started in India in 1952 with the establishment of a plant for the production of BHC near Calcutta, and India is now the second largest manufacturer of pesticides in Asia after China and ranks twelfth globally (Mathur, 1999). There has been a steady growth in the production of technical grade pesticides in India, from 5,000 metric tons in 1958 to 102,240 metric tons in 1998. In 1996–97 the demand for pesticides in terms of value was estimated to be around Rs. 22 billion (USD 0.5 billion), which is about 2% of the total world market.

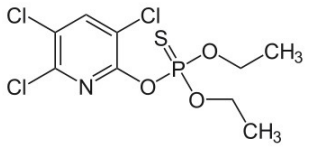
Chlorpyrifos industry has developed fast in the past years worldwide, especially in some developing countries, such as China and India. The demand for chlorpyrifos has witnessed rapid growth in many countries in recent years, which has promoted its large capacity expansion and

technology innovation, resulting in supply surplus as well as production cost decrease (CCM press release, China, 2011).

Chlorpyrifos:

Chlorpyrifos is an organophosphate insecticide. It is no longer registered for use inside homes except as bait in containers, but it is still used in agriculture, on golf courses, and to control public health pests such as fire ants. Chlorpyrifos blocks an enzyme that stops nerve cells from firing without stopping. If the enzyme is blocked, the nerves don't send normal signals, and the nervous system fails. Chlorpyrifos is used in agriculture, but it is no longer registered for residential use (Anuja, 2005). Chlorpyrifos belongs to category of Organophosphate pesticides which are generally regarded as safe for use on crops due to their relatively fast degradation rate, which varies as a function of microbial components, pH, temperature, hydrolysis, photolysis and other factors. Hydrolysis half-life of an OP pesticide of 10 days in the lab increases to one year if the pH is 6 and temperature is 5°C (Ragnarsdottiv, 2000). In one of the studies organic carbon content and relatively low pH of the soil greatly affected the released residue of chlorpyrifos; sterilization greatly reduced the rate of chlorpyrifos degradation indicating the involvement of microbial activities (Rehman and Motoyama, 2000).

Chlorpyrifos is the second largest selling OP agro chemical in India. Dursban is the most toxic organophosphorus compound and is more toxic than organochlorine compounds (Veeraiah *et al.,* 2001).



Structure of chlorpyrifos

The physical and chemical properties of a compound determine its behaviour in the environment. Although environmental variables are important in modulating transformation and transport processes, the basic pattern of persistence and partitioning is fundamentally derived from the chemical character of the compound.

The following are the characteristics of chlorpyriphos.

Common name: Chlorpyriphos
IUPAC name: O, O-diethyl O-3, 5, 6-trichloro-2-pyridyl phosphorothioate
Empirical formula: $C_9H_{11}Cl_3NO_3PS$
Molecular weight: 350.6
Colour: White/colourless crystalline solid (pure active constituent)
White to light yellowish-brown crystalline solid (technical grade)
Odour: Odourless
Melting point: 41 to 43.5°C
Vapour pressure: 1.87 x 10-5 mm Hg at 25°C
Solubility in water: 1.2 mg/L at 25°C
Solvent solubility (g/100 g) - Acetone: 650
Chloroform: 630
Benzene: 790
Diethyl ether: 510
Stability: Stable in air (non-volatile) and is not sensitive to UV radiation. It is stable to neutral and weakly acidic solutions, but is hydrolysed by strong bases.

(Source: Anuja, 2005)

Following table enlists the crops for which Chlorpyrifos is used and the target pests against which chlorpyrifos acts.

CROP	COMMON NAME OF THE PEST	DOSAGE/Ha OF CHLORPYRIFOS 20% EC		
		gm	Formulation (gm/ml)	Dilution in water (litres)
Beans	Pod Borer	600	3000	500-1000
	Black Bug	600	3000	500-1000
Gram	Cut worm	500	2500	500-1000

Sugarcane	Black bug Early shoot and stalk borer Pyrilla	150 250-300 300	750 1250-1500 1500	500-1000 500-1000 500-1000
Groundnut	Aphid Root grub	200 225	1000 1125	500-1000 500-1000
Mustard	Aphids	100	500	500-1000
Brinjal	Shoot and fruit borer	200	1000	500-1000

(CIBRC, 2009)

Toxicity and mode of action:

The chlorpyrifos toxicity can be broadly classified into two groups:

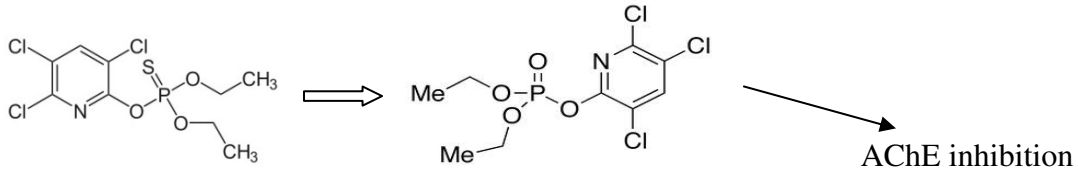
Chronic toxicity: Repeated or prolonged exposure to organophosphates may result in the same effects as acute exposure including the delayed symptoms. Other effects reported in workers repeatedly exposed include impaired memory and concentration, disorientation, severe depressions, irritability, confusion, headache, speech difficulties, delayed reaction times, nightmares, sleepwalking, and drowsiness or insomnia. An influenza-like condition with headache, nausea, weakness, loss of appetite, and malaise has also been reported. (US PHS 1989) When technical chlorpyrifos was fed to dogs for 2 years, increased liver weight occurred at 3.0 mg/kg/day. Signs of cholinesterase inhibition occurred at 1 mg/kg/day. Rats and mice given technical chlorpyrifos in the diet for 104 weeks showed no adverse effects other than cholinesterase inhibition (US EPA 1989).

Acute toxicity: Chlorpyrifos is moderately toxic to humans. (US EPA 1989) Poisoning from chlorpyrifos may affect the central nervous system, the cardiovascular system, and the respiratory system. It is also a skin and eye irritant. While some organophosphates are readily absorbed through the skin, studies in humans suggest that skin absorption of chlorpyrifos is limited (Gallo and Lawryk, 1991). Symptoms of acute exposure to organophosphate or

cholinesterase-inhibiting compounds may include the following: numbness, tingling sensations, in coordination, headache, dizziness, tremor, nausea, abdominal cramps, sweating, blurred vision, difficulty breathing or respiratory depression, and slow heartbeat. Very high doses may result in unconsciousness, incontinence, and convulsions or fatality (Gallo, 1991).

Chlorpyriphos applied topically to fire ants (*Solenopsis richteri*) is rapidly transported from the external cuticular surface to the hemolymph and organs, and greater than 90 per cent of the applied dose has been reported to get absorbed within 4 hrs (Chambers *et al*., 1983).

Chlorpyrifos is directly toxic to the nervous system. In addition, it is transformed inside animals to chlorpyrifos-oxon which is about 3000 times more potent against the nervous system as compared to chlorpyrifos itself (Goodman L.R., *et al.* 1985).



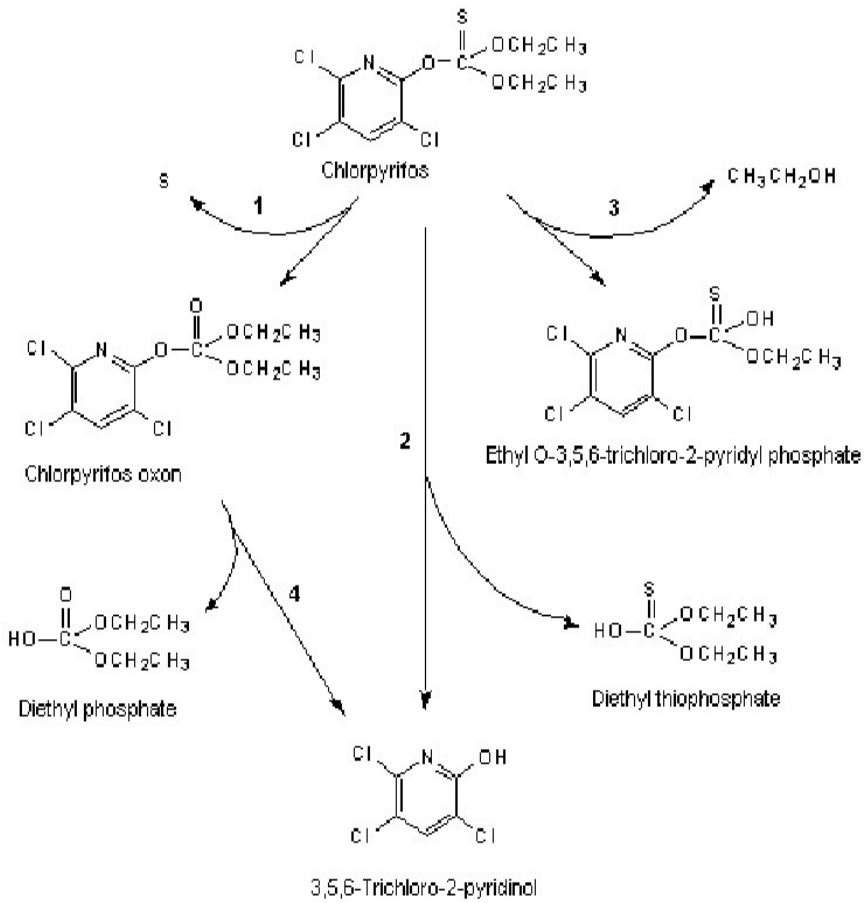
Chlorpyrifos chlorpyrifos-oxon

The toxicity of insecticidal organophosphorus and carbamate esters to animals is attributed to their ability to inhibit acetylcholinesterase (AChE, choline hydrolase, EC 3.1.1.7), which is a class of enzymes that catalyzes the hydrolysis of the neuro-transmitting agent acetylcholine (ACh). The inhibition of AChE and related esterases, often referred to as serine hydrolases, has been clearly demonstrated to be the result of an actual chemical reaction between the enzyme and the organophosphate or carbamate ester. The phosphorylated or carbamylated enzyme is no longer capable of affecting the hydrolysis of ACh; this results in a build-up of the neurotransmitter at a nerve synapse or neuromuscular junction (Fukuto, 1990).

OP pesticides acts by inhibiting acetylcholinesterase by phosphorylation and elicit a spectrum of cholinergic effects. The primary mechanism of toxicity for organophosphorus pesticides, like chlorpyrifos, is cholinesterase (ChE) inhibition. The inhibition of the enzyme acetylcholinesterase (AChE) results in the buildup of acetylcholine (ACh) at choline receptors, causing continual nerve stimulation (Giesy *et al.,* 1999).

Kanekar *et al*. (2004) has also reported that on exposure to organophosphates acetylcholinesterase enzyme is not able to function, causing accumulation of acetylcholine, which interfere with the transmission of nerve impulses at the nerve endings.

Chlorpyrifos is a relatively weak AChE inhibitor compared to its metabolite chlorpyrifos oxon (El- Merhibi *et al,* 2004), thus toxicity is initiated by the formation of chlorpyrifos oxon by oxidative desulfuration (Giesy *et al.* 1999). Factors influencing the toxicity of chlorpyrifos between species and groups include metabolic rate, the number of target sites available for chlorpyrifos metabolism to chlorpyrifos oxon (Chambers and Carr 1995), organism surface area, and lifestage (El-Merhibi *et al.* 2004).



Metabolic pathway of chlorpyrifos (Dhanya M.S., 2014)

Bioremediation of Chlorpyrifos:

Bioremediation which includes the gainful utilization of microorganism for the metabolism or biodegradation of target pollutants into safer and innocuous products is amongst the potent technologies that are being used globally for the restoration of contaminated sites (Anuja, 2005). Bioremediation is the process which utilizes naturally occurring microorganisms to breakdown hazardous substances unto less toxic substances. It uses relatively low-cost, low technology techniques, which generally have a high public acceptance and can be carried out on site (EPA).

Due to the ill effects of Chlorpyrifos on environment and growing toxicity, the need was generated to degrade the residues of chlorpyrifos to less harmful products. Many researches were done to find the efficient ways of degradation including chemical degradation.

Chemical degradation of pesticides have been reported by several researchers with 3, 5, 6-trichloro-2-pyridinol (TCP) as the intermediate compound. Chemical degradation has its own limitations as it does not completely degrades the TCP which is formed as an intermediate, it persists in soil even after degradation and it is a stable product. Thus, we need to find out ways where we can achieve complete degradation of chlorpyrifos.

Unlike in the case of other organophosphates, however, there have been no reports of enhanced microbial degradation of chlorpyrifos since its first use in 1965 until, recently when in a study by Feng and his co-workers in 1997, a pure culture of bacteria capable of using 3, 5, 6-trichloro-2-pyridinol (TCP) as the sole source of carbon and energy under aerobic conditions was isolated. Mallick *et al*. 1999, reported the rapid degradation of chlorpyrifos, added to a mineral salt medium as a sole carbon source by *Flavobacterium* sp. and *Arthrobacter* sp.
Huang *et al*. (2000) reported the degradation of chlorpyrifos by aerobic microbial processes in poultry and cow-derived effluents. Further, the microbes involved in the degradation process were analysed by denaturing gradient gel electrophoresis (DGGE) of PCR amplified 16sRNA genes and it was observed that a single band became dominant in effluents during chlorpyrifos degradation, which indicated that a single aerobic-bacterial population was capable of degrading chlorpyrifos.

43 cultures of bacteria from various soil samples were collected from different regions of Punjab and screened for the degradation of chlorpyrifos by Kahlon and Saurabh (2003). Four isolates were found to grow efficiently which were further selected for the bioaugmentation of pesticide degradation in the contaminated soil.

Singh *et al*. (2004) worked on biodegradation of chlorpyrifos by *Enterobacter* strain B-14 and its use in bioremediation. It was found that the addition of strain B-14 (106 cells/g) to soil inoculated with chlorpyrifos degrading bacteria, treated with 35 mg of chlorpyrifos/kg resulted in a higher degradation rate.

Yang *et al*. 2005 isolated *Alcaligenes faecalis* DSP3, which is capable of degrading chlorpyrifos and 3, 5, 6-trichloro-2-pyridinol (TCP).

Agus Sabdono (2007) found that a bacterium which utilizes chlorpyrifos as a sole source of carbon and energy isolated from coral surface utilized chlorpyrifos up to 25 mg l^{-1}. This strain demonstrated greatest similarity to members of the order *Bacillales* and was closest to members of the *Bacillus firmus* group.

Pseudomonas aeruginosa, common gram negative bacterium found in soil, has been found to have potential to degrade chlorpyrifos (Fulekar & Geetha 2008).

Five microbial consortia from chlorpyriphos contaminated sites by selective enrichment techniques were isolated by Khanna *et al.,* (2009). Out of them two were the promising consortia with degradation efficiency of 72 and 70 per cent respectively.

Zhiyuan Liu *et al*. (2012) worked on the bacterial degradation of chlorpyrifos in liquid medium and found that it degraded under the condition of 30˚C, pH 7.0, concentration of chlorpyrifos below 150 mg L^{-1}, with a degradation rate up to 78.85%.

G. Briceno *et al*. (2013) studied about the enhanced removal of chlorpyrifos and pentachlorophenol by single cultures and consortia of free and immobilized *Streptomyces* strains. The antagonism between the strains and their tolerance to the pesticides was evaluated. Results

obtained showed high tolerance levels against these pesticides and no antagonistic effects were seen. *Streptomyces* sp. M7 showed high degradation of chlorpyrifos (99.2%) and pure culture of *Streptomyces* sp. A5 showed degradation rate of 10.6% for pentachlorophenol.

Paenibacillus polymyxa and *Azospirillum* were reported to degrade organophosphorous pesticides-Chlorpyrifos, Chlorpyrifos-methyl and cyanophos in mineral salt medium. The pesticides were used as sole carbon and phosphorus source and it was also found that *Paenibacillus* was more effective than *Azospirillum* in degrading these pesticides (Romeh *et al.* 2014).

Plant growth promoting activity by pesticide degrading bacteria:

The inoculation of plants with beneficial bacteria to increase plant growth and protect plants form diseases is a promising strategy in modern crop production systems. Among bacterial inoculants are plant growth promoting rhizobacteria (PGPR), which are free living rhizosphere bacteria that promote plant growth and development by several mechanisms (Myresiotis *et al.*, 2014).

Direct plant growth promotion by PGPR involves the stimulation of root growth by producing plant hormones or facilitating the uptake of certain nutrients from the soil by various mechanisms such as nitrogen fixation, phosphate solubilization and synthesis of siderophores. They also include the production of plant hormones like auxins, gibberlins and cytokinins. PGPR can also indirectly induce plant growth by protecting plants against pathogens, a process known as biocontrol activity (Myresiotis *et al.*, 2014).

The inoculation of crops with PGPR may constitute an alternative way to increase productivity in a sustainable manner, reducing the use of agrochemicals which often pollute the environment. A number of studies have shown that PGPR are potential growth enhancers in several crops. In particular, successful examples of inoculation of corn, wheat, tomato and other crops with PGPR strains of the genera *Azospirillum, Bacillus, Pseudomonas* and *Enterobacter* have been achieved in laboratory and field experiments (Myresiotis *et al.*, 2014).

Strains of *Gordonia, Klebsiella, Paenibacillus, Serratia* are among others. *Bacillus* and *Pseudomonas* spp. have a wide distribution and are most extensively studied. In recent years, several PGPR-based products became commercially available in many countries, and many more are currently under development (Choudhary and Johri 2009).

Among the bacilli, strains of *Bacillus subtilis* are the most widely used PGPR because they are able to produce a broad spectrum of antibiotics, and effectively colonize the rhizosphere and control soil borne pathogens (Cazorla *et al.,* 2007).

Pseudomonas, Azospirillum, Agrobacterium, Bacillus, Enterobacter and *Flavobacterium* are some of the genera which include PGPR strains able to degrade organic and inorganic contaminants in soil (Zhuang *et al.,* 2007). In addition to biopesticides, many soil applied synthetic pesticides are used against weeds, pests and pathogens during conventional or integrated crop production programs. Furthermore, the attention has been paid on bioremediation of contaminated soils with PGPR (Jiang *et al.,* 2008).

Chlorpyrifos degradation is of high importance as it shows high mammalian toxicity. *Pseudomonas aeruginosa DKC2* showed good growth in basal salt medium suggesting their ability to use chlorpyrifos as sole source for carbon and energy. It also showed various plant promoting activities like phosphate solubilization, protease activity and Indole-3-acetic acid production (Bhatia *et al.,* 2013).

Acinetobacter calcoaceticus was isolated from rhizosphere contaminated with chlorpyrifos. The bacteria was capable of utilizing chlorpyrifos as the sole source of carbon for growth and it also showed multiple plant growth promoting traits of phosphate solubilization, Indole-3-acetic acid and siderophore production (Lei *et al.,* 2014).

yFp tagged *Mesorhizobium sp. HN3* was shown to efficiently colonize the rhizosphere of ryegrass planted in chlorpyrifos contaminated soil. It showed enhanced plant growth and degradation of chlorpyrifos and its metabolite 3, 5, 6 trichloro-2-pyridinol (TCP). Significantly lower residues of the pesticides were observed in plants grown in inoculated soil which might be attributed to the efficient root colonization of HN3yFp, suggesting its involvement in chlorpyrifos degradation inside the roots and rhizosphere of plants (Jabeen *et al.,* 2015).

A recent study was done by Pratibha Yadav *et al*. (2015), on remediation of organophosphate pesticide Methyl parathion using 4 Rhizobacteria isolates- RB1, RB2, RB3 and RB4. These isolates were grown on a medium containing methyl parathion as sole carbon and/or nitrogen source. RB1 and RB4 were found to have maximum enzyme activity for degradation of methyl parathion. Plant bioassays were also performed where on inoculation with these isolates the plant growth was observed in terms of leaf count, seed vigour, and growth increment in radical length and percentage increment in seed germination.

Shamsa Akbar *et al.* (2016) isolated two bacterial strains JCp4 and FCp1 having chlorpyrifos degradation potentials of 84.4% and 78.8% respectively. These strains were identified as *Achromobacter xylosoxidans* (JCp4) and *Ochrobactrum sp.* (FCp1) based on 16s rRNA sequence analysis. These strains also showed plant growth promoting activities like indole acetic acid production and phosphate solubilization in both absence as well as presence of chlorpyrifos. In plant growth experiments conducted on *Vigna unguiculata*, it was found that these strains enhance plant growth significantly in terms of plant weight and plant length and it was noted that strains shows increased degradation rate of chlorpyrifos in planted soil.

OBJECTIVES

The objectives of the study are:

1. To isolate the microorganisms capable of degrading chlorpyrifos present in the soil.
2. Biochemical, morphological and molecular characterization of the isolated microorganism.
3. Study of the plant growth promoting efficacy of the characterized microorganism.
4. Study of the interaction between isolated microorganism and *Pennisetum glaucum.*

PROPOSED METHODOLOGY

Soil sampling:

The soil samples will be collected from various fields of Jaipur and nearby villages. The soil samples will be of two types: soil without pesticide treatment and soil pretreated with chlorpyrifos.

Topsoil will be collected and stored in polythene bags for the development of aerobic microbial consortia utilizing chlorpyrifos as sole carbon and energy source, by enrichment process (Vidyalakshmi, 2010).

Soil parameters:

The properties of soil like organic matter, pH, clay content etc. affect the degradation of pesticide in soil, which makes its study a very important aspect as described by Gold *et al.* (1996).

The effects of pH, soil moisture, organic matter content, soil salinity are some parameters that would be studied to understand their effects on degradation of chlorpyrifos.

Pesticide:

The technical grade Chlorpyrifos will be purchased from a pesticide manufacturer.

Preparation of soil sample:

The soil sample will be prepared by drying it at room temperature, thorough mixing and then sieving through 2mm sieve. The sieved soil will be then stored at 4°C. In 50 grams of soil, 1ml of pesticide will be added and will be incubated for 24 hours at 37ºC, for the enrichment of native microflora.

Isolation of bacterial colonies:

The native bacteria of the soil will be maintained on the minimal salt medium before being assessed for their biodegradative properties against chlorpyrifos. Then, the serial dilutions of bacterial cultures will be prepared. These serially diluted bacterial cultures will be tested on MSM plates. The carbon source in MSM media will be replaced by chlorpyrifos. Different

concentrations of pesticide ranging from 10 to 32 mg L^{-1}will be added and growth of bacteria will be checked on these plates by incubating them for 3 days at 37°C.

The bacterial cultures will also be tested for showing maximum tolerance levels (MTL) by inoculating cultures on MSM plates having highest pesticide concentration (32mg/L^{-1}). The colonies showing growth will have maximum tolerance levels and these colonies will be further taken for preparation of mother culture. The colonies from mother culture will be taken for characterization of bacteria.

Degradation Studies:

The estimation of degraded chlorpyrifos in bacterial strains will be carried out using High Performance Liquid Chromatography (HPLC). For determining the degradation potential, cultures grown in MSM will be taken and the residual chlorpyrifos concentration will be determined by high performance liquid chromatography (HPLC). Chlorpyrifos quantification will be analyzed using an S1122 HPLC Pump, S3210 UV detector, S1122 delivery system and Phenomenex C18 reversed-phase column (150mm). Samples will be eluted using methanol: H2O: acetic acid (80:20:0.5, v/v) at a flow rate of 1.0mL min^{-1}. The bacterial strains will be outsourced for conducting the HPLC analysis.

Characterization of Bacteria:

The isolated bacterial colonies will be characterized. Morphological characters viz. size, shape will be determined by visual observation as well as by using light trans-illuminator and microscopy. The Gram staining technique will be used for differentiation between gram positive and gram negative bacterial strains.

Further the biochemical characterization of bacteria will be done. The biochemical tests that will be performed are listed in the table 1. These biochemical tests are performed by the process given in *Microbiology: A Laboratory manual by Cappuccino and Sherman*.

S.no	Test
1.	MRVP test
2.	Nitrate reduction test

3.	Catalase test
4.	Oxidase test
5.	Gelatinase test
6.	Malonate utilization test
7.	D-arabinose test
8.	Urease test
9.	Indole test
10.	D-gluconic test
11.	Citrate utilization test

16s rRNA sequencing:

The bacterial strains will be further taken for molecular characterization by 16s rRNA sequence analysis. The genomic DNA will be isolated from bacterial cell using standard CTAB method. The 16s rRNA gene will be amplified by PCR using universal primers 5'-AGAGTRTGATCMTYGTCWAC-3' and 5'-CYGTAMCTTWTTAGCRCT-3' and the purified PCR product will be outsourced for sequencing of both strands, as described by Das *et al.* (2012).

Determination of optimum pH and temperature conditions:

The bacterial strains growing on pesticide supplemented medium will be tested for their growth in different pH conditions. This will be done by preparing agar plates having set on different pH ranges and then inoculating bacteria on those plates. These plates will incubated at 28°C for 4 days and growth of bacteria will be observed and the optimum pH value will be determined.

Similarly, optimum temperature condition will be determined by incubating the inoculated plates at different temperature ranges so as to determine the temperature that supports the maximum growth of bacteria.

Study of growth kinetics of chlorpyrifos degrading bacteria:

The cultures of chlorpyrifos degrading bacteria will be inoculated in a flask having 100ml of media. These flasks will be incubated at 37°C in a shaking incubator and O.D. at 540nm will be recorded periodically.

Again, the same process will be repeated to understand the growth kinetics of bacteria in the presence of pesticides. The flask with 100ml of media will then be supplemented with chlorpyrifos and the inoculated bacteria will be incubated.

Plant microbe interaction:

When the isolated bacteria is inoculated in soil treated with pesticide, we can test whether the bacteria is capable of not only degrading the pesticide in soil but also affects the growth of crop plant growing in that soil. Plant microbe interaction will be studied by conducting pot experiments with *Pennisetum glaucum* to study the effects of bacterial inoculation on plant growth and pesticide degradation. The seeds of the plant will be surface sterilized by treating with 0.1% $HgCl_2$ solution for 5 minutes followed by washing with sterilized distilled water. Chlorpyrifos with a concentration of 200 mg kg^{-1} will be added to soil samples which further will be inoculated with isolated microorganisms. Surface sterilized seeds will be sown in soil samples and proper care of moisture, light and temperature will be taken until seed emergence process is observed.

Analysis of PGPR activity:

Isolation and screening of Phosphate-solubilizing bacteria:

To isolate phosphate solubilizing bactetia from soil samples collected, a serial dilution assay will be carried out in 0.9% NaCl solution. 10µL of diluted suspension will be inoculated on Pikovaskya agar medium by spread plate technique and the agar plate will be incubated at 28°C for 7 days.

The isolates showing clear halo around bacterial colonies after incubation period will be considered as P-solubilizing bacteria. Further these bacterial isolates will also be tested for their phosphate solubilizing activity in presence of chlorpyrifos by adding pesticides in Pikovaskya

medium and then incubating the inoculated agar plates at 28°C for 7 days. Again, the bacterial colonies showing clear halo around them will be selected.

The extent of P-solubilization will be determined in Pikovaskya broth by chlorostannous-reduced molybdophosphoric acid blue method (King, 1932; Jackson, 1967).

Indole acetic acid production:

The Indole acetic acid production will be quantitatively analyzed by method of Gordon and Weber (1951). The bacterial strains will be grown on LB medium having fixed concentration of tryptophan. Plates having different pesticide concentrations will be prepared and 0.1ml of culture will be inoculated. The plates will be kept for incubation for 7 days at 28°C with shaking at 125rpm.

After 7 days, 5 ml culture will be centrifuged at 9000g for 15 minutes and an aliquot of 2ml supernatant will be mixed with 100µL of orthophosphoric acid and 4 mL of Salkowsky reagent and incubated at 28°C in darkness for 1 hour. The absorbance will be read at 530nm and IAA concentration will be determined by using calibration curve of pure IAA as standard.

Siderophore production:

The siderophore production by bacterial colonies will be analyzed by supplementing Chrome azurol S (CAS) agar plates with pesticide. The plates will be spot inoculated with 10µL of 10^8 cells mL^{-1} and will be kept for incubation at 28°C for 4 days. Development of yellow to orange halo around bacterial colonies will indicate siderophore production.

SIGNIFICANCE

Green revolution has marked its importance in India with increased crop production. With increased demand for crops, the consumption of pesticides to kill pests has increased several folds. The widespread use of pesticides is a necessity as a farmer has to bear severe loss because of the destruction caused by pests. The use of pesticides has resulted in problems, caused by their interaction with the biological systems in the environment as they harm soil microorganisms as well as get accumulated in the soil.

Considering the toxic effects of pesticides, it is essential to remove them from the environment. Several methods of pesticide degradation have been employed to remove them from the environment, including chemical methods, but their use made the situation even worse. It is of much importance to make use of biological methods to combat the situation which gives rise to bioremediation. It is the process which makes use of microorganisms which uses xenobiotic compounds like pesticides for their growth, mineralize and detoxify them.

Chlorpyrifos is an organophosphate pesticide, acutely toxic to humans and harms the soil microflora when get accumulated. It influences the soil microbial activities when applied to it. A modular approach to degrade these pesticides is very essential and use of microorganisms can be one such approach. Factors like temperature, pH, and available sources of Nitrogen and phosphorus influence the microbes to co-metabolize the pesticide. Therefore, we can say that bioremediation is nature's way of recycling waste and then their reuse by the organisms.

Various bacterial and fungal species have been reported to be able to grow on chlorpyrifos such as *Serratia* sp. and *Pseudomonas* sp. (Cycon *et al.,* 2009), *Paracoccus* sp. and *Enterobacter* strain B-14 (Rani *et al.,* 2008; Xu *et al.,* 2008; Singh *et al.,* 2004; Li *et al.,* 2007; Bhagobaty and Malik, 2008). These bacteria use chlorpyrifos as their sole carbon and energy source thereby reducing their concentration in soil. Bacteria play an important role in degradation due to hydrolytic and oxidative enzymes. It is a viable option for cleaning the pesticide contaminated site as it is the most eco-friendly, highly efficient and cost effective method.

The intermediate metabolite of chlorpyrifos, 3, 5, 6-trichloro-2-pyridinol (TCP) also requires complete degradation as it is toxic. *Alcaligenes faecalis* DSP3, *Sphingobacterium* sp. JAS3, *Ganoderma* sp. JAS4, *Gordonia* sp. JAAS1 were a few microbes that mineralize and degrade the chlorpyrifos and its intermediate TCP (Yang *et al.,* 2005; Silambarasan *et al.,* 2013; Abraham *et al.,* 2013).

Besides pesticides another problem that farmers face is continuous use of fertilizers to meet the increasing demand of crops. Fertilizers increase the yield of crops by providing the required nutrients in optimum amounts to the crop plants. Certainly these fertilizers are the chemical substances which accumulate in the soil and are harmful for environment as they have high biological persistence.

Plant growth promoting activity has been shown by certain chlorpyrifos degrading bacteria and has been reported by various researchers. Bacteria like *Pseudomonas aeruginosa* DKC2, *Acinetobacter calcoaceticus* and yFp tagged *Mesorhizobium sp.* HN3 have shown potent pesticide degrading capability along with plant growth promoting activities.

If the bacteria that degrade chlorpyrifos can also secrete certain metabolites that can promote the plant growth then these bacteria can also act as bio-fertilizers which can be a boon for farmers. This will reduce their dependency on chemical fertilizers without compromising with the high yields of crop plants.

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